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KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

GRADIPORE LIMITED

PROVISIONAL SPECIFICATION

Invention Title:

Improved blood coagulation test

The invention is described in the following statement:

Technical Field

The present invention relates to an improved test for measuring blood coagulation potential of patients' plasmas.

Background Art

Mechanisms for blood coagulation, thrombosis and haemostasis are well described in International Patent Publication WO 91/01382 the contents of which are incorporated herein by reference.

It is known from International Patent Publication WO 93/01261 and publications by Bertina et al 1994 and Dahlback et al 1995 that the risk of thrombosis in patients with a mutant factor V molecule known as the Leiden variant, or with activated protein C impairment for some other reason, may be determined by activating the coagulation system in a plasma sample and incubating the sample with activated protein C in what has come to be known as an activated protein C impairment, impedance or resistance test. There are precedents for this test in which impairment of activated protein C has been detected in patents with acquired thrombophilia (Mitchell et al, 1986; Amer et al, 1988).

New tests have recently been proposed to screen for all defects in the protein C pathway (PCP) and thereby to rationalise the approach to individual assays for protein C, S and factor V (Leiden). These tests usually involve activating the patient's own protein C either with thrombin/thrombomodulin or the activator from *Agkistrodon Contortrix* venom. This activated protein C (APC) then inactivates the patient's own factor V in a protein S-dependent manner. Thus clotting times shorter than normal are obtained when defects in protein C and protein S occur as well as when APC resistant factor V (Leiden) is present. Such tests have been described based on Activated Partial Thromboplastin Times (APTT) eg AU 28416/95 and on dilute prothrombin times tests (PT).

A substrate conversion reaction rate may be determined by the coagulation time or by the time required for the conversion of a chromogenic substrate to a coloured product. The conversion rate obtained is compared with values obtained in the absence of APC and also with results for normal plasma samples. If the coagulation time is not sufficiently prolonged by APC, it indicates that the individual from which the sample is derived may be at a higher-than-normal risk of thrombosis.

It is well known that activation of endogenous protein C in plasma by the activator from *A. Contortrix* venom prolongs subsequent clotting times to a degree related to the protein C content. Several other factors, however, influence or interfere with this test. These factors include protein S, factor V (Leiden) and now recognised as thrombotic risk factors in their own right.

The present inventor has recently developed an improved APC resistance test which is described in WO 96/04560. This test requires the addition of exogenous reagents which activate factor V and activate the common pathway of the blood coagulation mechanism through factor X or by inducing the formation of thrombin in a factor V dependant manner together with exogenous APC to a plasma sample. It was found that if factor V is specifically activated by an exogenous reagent in addition to activation of the common pathway through factor X, the test for APC resistance may be made more sensitive and specific than previously known tests. The present inventor has also found that improved specificity is obtained when a complex factor X activator is used together with the factor V activator. This test has been referred to as the Russell Viper Venom (RVV) -based test. A similar result is achieved if prothrombin is activated to thrombin by a factor V dependent activator in the presence of a factor V activator.

The protein C pathway is one of a number of antithrombotic mechanisms operating within normal blood vessels to control coagulation. Probably the most important of these mechanisms is the glycoaminoglycan (GAG) pathway which requires antithrombin III as a cofactor.

The present inventor has made the surprising finding that such tests may be further modified to allow improved discrimination between healthy individuals and patients with impaired or aberrant blood anti-thrombotic mechanisms.

Disclosure of Invention

In a first aspect, the present invention consists in a method for determining the coagulation potential of a plasma sample comprising the steps of:

(a) pre-incubating the plasma sample with a reagent such that (1) endogenous protein C in the plasma is converted into activated protein C by the reagent; (2) adding to the pre-incubated plasma sample an activator of factor V, and (3) adding an activator of factor X or factor X;

(b) adding to the pre-incubated plasma sample mixture (a) a reagent to initiate clotting comprising:

(i) an exogenous reagent which activates the common pathway of the blood coagulation mechanism through factor X or by inducing the presence of thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the potential rate of coagulation of the plasma sample; and

(d) comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient, or comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for the plasma sample in the absence of protein C activator, and determining the coagulation potential of the plasma sample from one or other of those comparisons.

The reagent used in step (a) preferably also contains activated factor X and heparin. The inclusion of these components to the reagent makes the test sensitive to antithrombin III.

Preferably, the exogenous reagent which transforms protein C into activated protein C is diluted substantially whole snake venom, preferably diluted snake venom from *Agkistrodon Contortrix*, or related species such as *A. Piscovorus, A. Bilineatus, A. C. Laticictus, A. C. Mocason*. It has been found that by selecting an appropriate concentration of the snake venom, it is possible to obtain a diagnosis of impaired coagulation by the one test. A protein C pathway (PCP) ratio of below a pre-determined value can be indicative of impaired coagulation in the patient's plasma. When using *A. Contortrix* whole venom diluted at a concentration of about 0.002%, it is possible to differentiate between normal sera, sera from pregnant individuals and sera containing exogenous coagulation inhibitors, from sera obtained from patients with coagulation abnormalities. A PCP ratio in this instance of below about 2 would be positive in the present test. Similarly for a concentration of 0.002%, a value of below 2.5 would be positive.

Preferably, the incubation in step (a) is carried out at neutral or slightly basic conditions, more preferably at about pH 7.5. The incubation is carried out for sufficient time for activation of the protein C in the plasma.

Typically incubation times of around 5 minutes have been found to be sufficient.

The present inventor has made the surprising finding that the protein C activator purified from *A. Contortrix* venom (a commercial product "Protac" available from Pentapharm AB (Switzerland)) does not work very well in the present invention. The present inventor has found that dilute *A. Contortrix* venom is particularly suitable. It is possible that the purification process used to produce this commercial protein C activator removes an additional activator or agent that is present in whole venom which is preferably required for the present invention. It will be appreciated that this additional activator or agent could also be purified from whole venom and combined with the commercially available purified protein C activator for use in the present invention. The individual active fractions may also be purified and recombined to produce a reagent suitable for the present invention.

Factor Xa of either human or animal origin can be included and incubated with the protein C activator reagent. This factor can be formed from endogenous factor X by venom activators. Also, heparin can be included in the reagent to enhance the interaction between antithrombin III and factor Xa to enhance sensitivity to low levels of antithrombin III.

In a preferred form of the present invention, the pre-incubated patient's plasma sample is incubated with an exogenous activator for factor V and factor X. The exogenous activators for both factors X and V are most preferably derived from snake venom. In one embodiment of the invention both the factor V and the factor X activators are derived from Russells viper venom (RVV). The factor X activator is preferably derived from the venom of Russell viper (*Vipera Russelli*) and other immunologically cross-reactive species. A preferred factor V activator is derived from *Naja Nivea* and other immunologically cross-reactive species. The snake venoms may either be used in a diluted but unfractionated form which contributes to the simplicity of the test or, preferably, may be used in a fractionated form utilising isolated venom components.

Rather than directly activating factor X with an exogenous reagent one may also obtain an improvement over the known activated protein C test by utilising an exogenous reagent that induces in the plasma the presence of thrombin in a factor V dependent manner. In this aspect of the invention factor V dependent prothrombin activators such as those from certain

Australian *Notechis* and *Pseudonaja* venoms, such as *Pseudonaja Textilis*, *Notechis Scutatus* and *Oxyuranus Scutellatus*, may be used. The use of this system by-passes factor X and all factors above it thereby making the test more specific than that based on Russells viper venom alone. The use of additional venom-derived factor V activators is desirable exactly as described above for the Russell viper venom activated system which involves factor X activation.

In one embodiment of the invention, the components in step (b) with which the patient's plasma and its pre-incubants are to be mixed are combined into a single mixture by the use of suitable surfactants, particularly non-ionic detergents. Such a single mixture preferably also contains supplemental components such as suitable buffers and preservatives. In addition the mixture preferably contains polybrene or another similar agent to reverse the effect of any heparin that may be present in the test samples. The incubation mixture preferably also contains relatively high levels of phospholipid at high ionic strength to overcome non-specific inhibitors such as lupus anticoagulants that may be present in the plasma sample.

Another complicating feature in test plasma samples may be the defect caused by oral anticoagulants. Many such patients may already be on oral anticoagulant treatment which affects the coagulation tests currently used to assess activated protein C resistance. The conventional method for minimising such interference is by mixing test plasmas with factor V deficient plasma. The present invention, however, does not necessarily require such manipulation as such inhibitors do not necessarily adversely effect the test.

The detection system for monitoring the potential rates of change within the coagulation system may be a coagulation time assay or a chromometric or fluorometric assay using an appropriate synthetic substrate. Such detection systems are well known and described in the patent specifications referred to in the introductory portions of this specification.

Some patients' plasmas may give borderline results when assayed by the method according to the present invention such that it is not possible to determine unequivocally between "normal" and factor V Leiden deficient serum samples. The present inventor has made the surprising discovery that diluting these "borderline" samples with low ionic strength solutions including water and carrying out the method according to the first aspect of

the present invention can differentiate between normal and factor V Leiden samples.

In a second aspect, the present invention consists in method to differentiate between patients with factor V Leiden from normal individuals, the method comprising diluting plasma from the patients and the normal individuals with low ionic strength solutions including water and repeating the method according to the first aspect of the present invention.

Preferably, the plasma are diluted 1:1 with water, preferably distilled or filtered water, prior to repeating the coagulation assay. The factor V Leiden plasma will usually have ratios equal to or less than the ratios obtained when undiluted. Furthermore, the ratios obtained for the factor V Leiden plasma will usually be less than the ratios obtained for normal plasma assayed with the same test conditions. Prior to the present invention, it would have been necessary to add factor V deficient plasma to all plasma test samples and then re-assay for clotting abnormalities.

The use of low ionic strength solutions, and particularly distilled water, is significantly cheaper than factor V deficient plasma that is required in tests currently used. Furthermore, low ionic strength solutions, and particularly distilled water, are far easier to source than factor V deficient plasma. The present invention therefore offers a real advantage in cost and availability over other tests requiring factor V deficient plasma presently in use.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

In order that the nature of the present invention may be more clearly understood, a preferred form will be described with reference to the following example and the accompanying drawings.

Brief Description of Drawings

Figure 1 shows results of varying *A. Contortrix* whole venom dilutions on different test plasmas; and

Figure 2 shows the results of re-assay of borderline samples to differentiate between normal and factor V Leiden plasma.

Modes for Carrying Out the Invention

METHOD

A method is described for a clotting test which is more specific for detecting resistance to activated protein C due to the factor V (Leiden) mutation than the original system described by Dahlback. The method involves 2 steps. In the fist step, test plasma is incubated with dilute whole *Agkistrodon Contortrix* venom at 0.002-0.004% and pH 7.5 for 5 minutes. In the second step, phospholipid-rich Russell viper venom is added and the time required for a fibrin clot to form is determined.

A "control" or blank test to detect baseline coagulation abnormalities may be carried out in exactly the same way, except that no *Agkistrodon Contortrix* venom should be present in the first pre-incubation step. Chromogenic substrates could be used as an alternative to clot formation for detecting the formation of thrombin, but these are more expensive.

Mechanism

It is known that *Agkistrodon Contortrix* venom contains an activator of protein C. The active component has been isolated and sold under the trade mark "Protac" by Pentapharm AB (Switzerland). Protac has been patented for use in tests for quantitating protein C and more recently in tests for assessing the function of the protein C pathway (PCP) as described above. During the course of the first incubation (above) protein C in the test plasma is converted to an enzymatically-active form (activated protein C or APC). This is a powerful anticoagulant which destroys factors Va and VIIIa, thereby interfering with the clotting mechanism and prolonging certain clotting tests. In individuals who are deficient in protein C or S, the anticoagulant effect of the venom protein C activator is reduced relative to normal and the clotting times are shorter than normal. Also, if the patient plasma contains a commonly-occurring mutation in factor V called the FV(Leiden) variant, the clotting times are less prolonged by either activated protein C or the venom protein C activator than with normal plasma. FV(Leiden) lacks a specific APC sensitive cleavage site involved in the inactivation of normal factor V and therefore it persists in such test systems and shortens the clotting times.

All of these defects interfere with the normal functioning of the PCP and are associated with clinical thrombosis. All three defects are usually detectable by a shorter than normal clotting test result in the presence of protein C activator. Patients who are on oral anticoagulants have reduced

levels of vitamin K-dependent clotting factors as well as protein C and S and cannot usually be screened for factor V(Leiden) which such a test. It has become conventional to mix such patient's plasmas with factor V deficient plasma to "correct" all clotting factor defects and protein C and S levels prior to carrying out an APC resistance tests for factor V(Leiden).

The present inventor has found that use of whole dilute *Agkistrodon Contortrix* venom (ACCV) is preferable to the use of isolated protein C activator in the RVV-based PCP test described in WO 96/04560 for the following reasons. The test becomes insensitive to protein S deficiency and less affected by low protein C levels. The effect of relatively high levels of ACCV on the RVVT of normal plasmas seems to be similar to that of lower levels, unlike that of the isolated activator which prolongs the RVVT to an increasing degree with concentration. Higher levels of ACCV can be used to activate the small concentrations of protein C found in patients on oral anticoagulants for more effect in the test and to overcome acquired APC resistance in patients taking oral contraceptives or who are pregnant. Thus by using higher levels of the whole ACCV it is possible to screen for the Factor V(Leiden) defect in plasmas from Warfarin patients, pregnancy plasmas and other conditions which previously required mixing with factor V deficient or other normalising factors.

Advantages

1. No need to mix test plasmas with factor V deficient plasma for the detection of factor V(Leiden) among complex patients.
2. Plateau concentration dependencies means higher levels of ACCV can be added with a less prolonged normal clotting time.

METHOD

The improved test is based on the factor V(Leiden)-specific PCP screening test which uses a phospholipid-rich RVV reagent. The composition of the reagent has been modified to make it less sensitive than usual to variations in Protein C and Protein S levels in the presence of a protein C activator. Since this RVV reagent is already designed to be heparin and lupus anticoagulant resistant and since its mechanism is through the common pathway, this test is more reliable than those based on APTTs and PTs

Reagents

1. Protein C Activator (PCA)

   Preparation

   - Reconstitute in volume of distilled water as indicated on the vial
   - Gently invert to mix- DO NOT shake
   - Allow to stand at room temperature for 10 minutes before use.

2. PRVV Reagent (Phospholipid-rich Russell viper venom reagent)

   Preparation

   - Reconstitute in volume of water as indicated on the vial
   - Gently invert to mix- DO NOT shake
   - Allow to stand at room temperature for 10 minutes before use.

Reconstituted Stability

Product	Conditions	Time
PCA	2-8°C	48 hours
	37°C	12 hours
PRVV	2-8°C	48 hours
	37°C	12 hours
	-20°C (freeze thaw only once)	1 month

Specimen

Mix nine parts of freshly collected blood with one part 3.5% (0.12 M) trisodium citrate. Centrifuge as soon as possible after collection at $\geq$ 1500 g for 15 minutes. Separate plasma and store at 2-8°C. Test within 4 hours of collection. Plasma may be stored frozen at -30°C or below for up to six months.

Jaundiced, lipaemic and haemolysed specimens can give false clotting time results. These results may also occur in patients with abnormal haematocrits, as plasma to citrate concentration in these samples is not optimal.

Test Procedure

Method

The PCP Test is not affected by Heparin levels of up to 0.5 IU/ml.

It is recommended that a 1:1 mix of patient plasma to factor V deficient plasma is used for testing of patients on oral anticoagulants. This will correct the factor deficiencies otherwise compromising the test.

Test with PC Activator

1. Pre-warm a slight excess of PRVV reagent, allowing 0.1 ml per test, to 37°C ± 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of Activator to the test plasma and warm at 37°C for 5 minutes.
4. Add 0.1 ml pre-warmed PRVV reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

Test without PC Activator

1. Pre-warm a slight excess of PRVV Reagent, allowing 0.1 ml per test, to 37°C ± 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of distilled water to the test plasma and warm at 37°C for 5 minutes.
3. Add 0.1 ml pre-warmed PRVV Reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

The results of PCP tests of varying *A. Contortrix* whole venom levels on various test plasmas are shown in Figure 1. The use of levels of between 0.002 to 0.004% ACCV in the test allows the differentiation between sera from normal individuals (PNP1, PNP2 and PNP3), oral anticoagulant pool (O/A pool) and pooled sera from pregnant individuals(PREG.POOL), from patients with impaired clotting function (FV(L) and FV(L)+O/A). PCP values of below 2 and 2.5, respectively for tests using 0.002 and 0.004% ACCV are seen to be indicative of impaired clotting.

Factor V Leiden Confirmation Assay

In order to differentiate between normal and factor V Leiden samples which gave "borderline" results in the blood clotting assay according to the present invention, samples were re-assayed after first being diluted 1:1 in distilled water. The results are shown in Figure 2. The three factor V Leiden samples tested produced lower ratios than those of the normal samples re-assayed after dilution with water. This finding allows the testing of samples without the need to add factor V deficient plasma to the samples to be assayed.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 2nd day of February 1998

GRADIPORE LIMITED
Patent Attorneys for the Applicant:

F.B. RICE & CO.

References

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DAHLBACK B - Inherited Thrombophilia: Resistance to activated protein C as a pathogenic factor of venous thromboembolism. Blood 85; 607-614, 1995

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AMER L, KISIEL W, SEARLES RP, WILLIAMS JRC - Impairment of the protein C anticoagulant pathway in a patient with systemic lupus erythematosus, anticardiolipin antibodies and thrombosis. Thromb. Res. 57, 247-1990

PCP TESTS WITH VARYING ACTIVATOR LEVELS ON VARIOUS TEST PLASMAS.

APTT-LIKE PCP TESTS USING REGULAR LA-CONFIRM AND 0.05M TRIS/HCL pH 7.4 BLANKS.

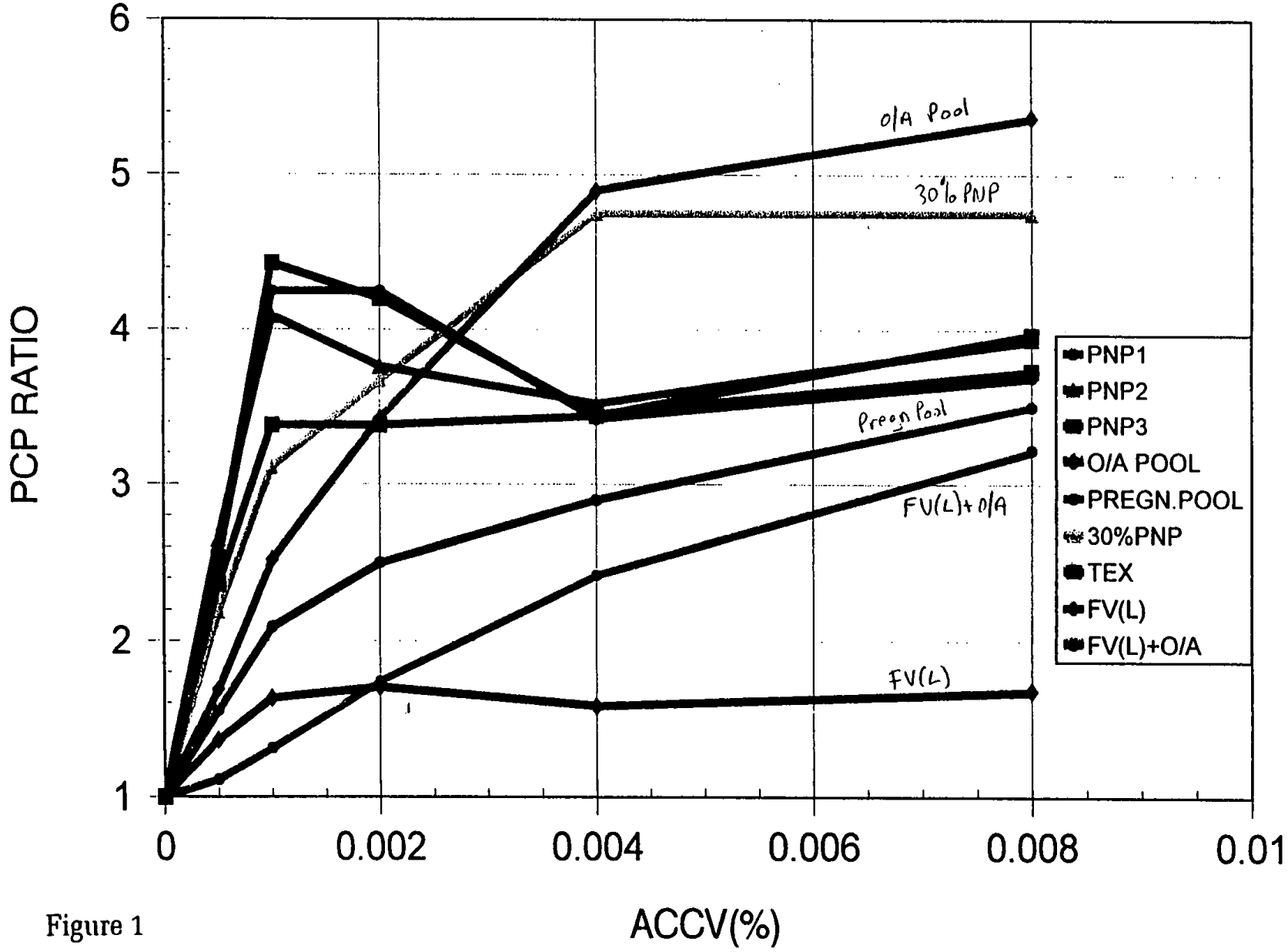

Figure 1

BORDERLINE FVLtest RESULTS II

Investigation of methods to resolve FVL cases from others among warfarin patients. All results on selected warfarin patients with initial borderline FVLtest results using 3 min.preincubation of samples with VCA on the ACL300.

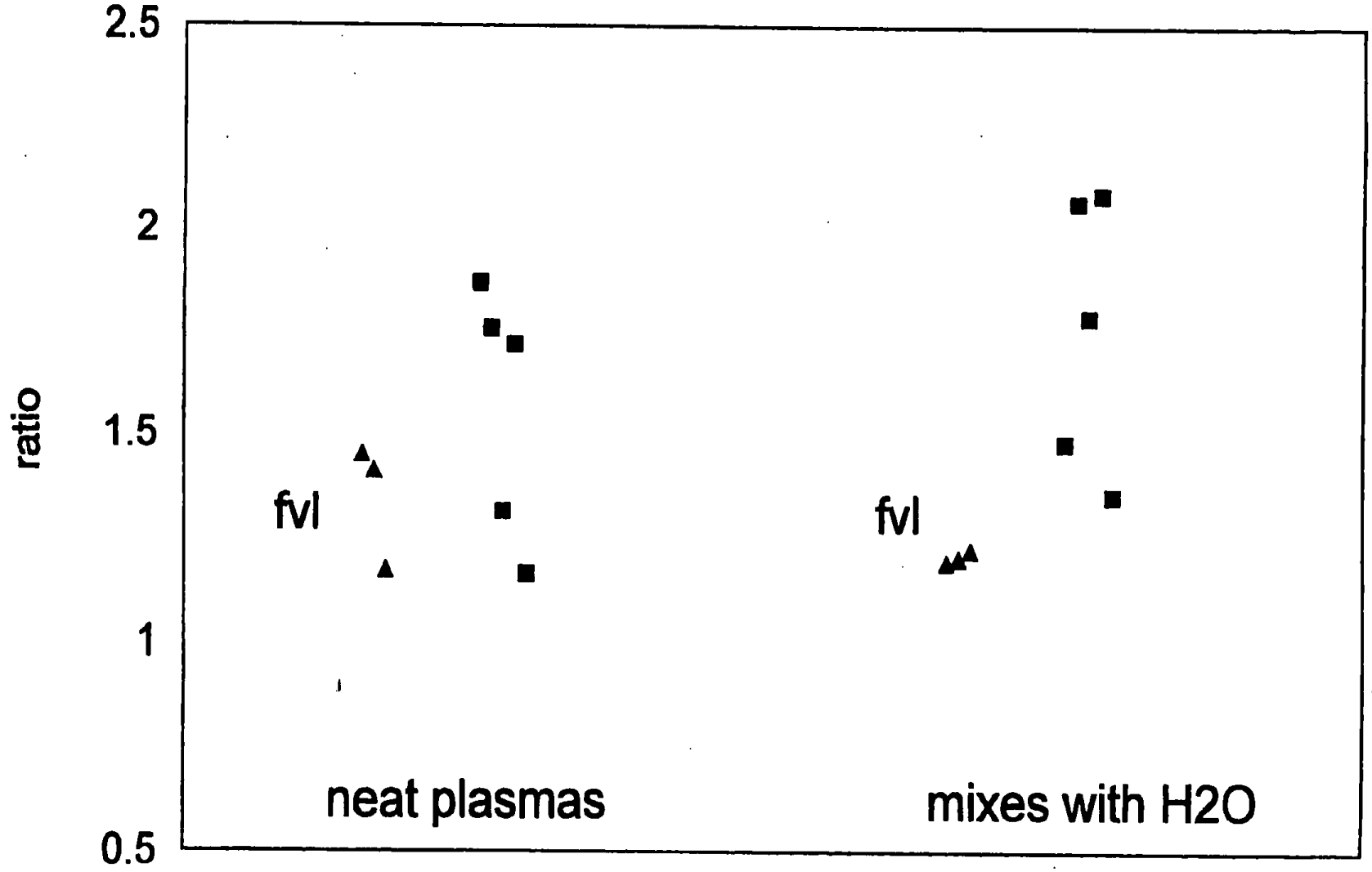

Figure 2